Enamel Matrix Protein Adsorption to Root Surfaces in the Presence or Absence of Human Blood

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Background: The clinical use of an enamel matrix derivative (EMD) has been shown to promote formation of new cementum, periodontal ligament (PDL), and bone and to significantly enhance the clinical outcomes after regenerative periodontal surgery. It is currently unknown to what extent the bleeding during periodontal surgery may compete with EMD adsorption to root surfaces. The aim of this study is to evaluate the effect of blood interactions on EMD adsorption to root surfaces mimicking various clinical settings and to test their ability to influence human PDL cell attachment and proliferation.

Methods: Teeth extracted for orthodontic reasons were subjected to ex vivo scaling and root planing and treated with 24% EDTA, EMD, and/or human blood in six clinically related settings to determine the ability of EMD to adsorb to root surfaces. Surfaces were analyzed for protein adsorption via scanning electron microscopy and immunohistochemical staining with an anti-EMD antibody. Primary human PDL cells were seeded on root surfaces and quantified for cell attachment and cell proliferation.

Results: Plasma proteins from blood samples altered the ability of EMD to adsorb to root surfaces on human teeth. Samples coated with EMD lacking blood demonstrated a consistent even layer of EMD adsorption to the root surface. In vitro experiments with PDL cells demonstrated improved cell attachment and proliferation in all samples coated with EMD (irrespective of EDTA) when compared to samples containing human blood.

Conclusion: Based on these findings, it is advised to minimize blood interactions during periodontal surgeries to allow better adsorption of EMD to root surfaces. J Periodontol 2012;83:885-892.

KEY WORDS
Dental cementum; enamel matrix derivative; periodontal ligament cells; periodontal regeneration; root planing; wound healing.

The goal of regenerative periodontal therapy is to restore the supporting structures of the tooth that have been lost after inflammatory periodontal disease and should result histologically in formation of new cementum, new periodontal ligament (PDL), and new bone (i.e., periodontal regeneration) over a root surface previously exposed to bacterial plaque.¹ One well-established method to enhance periodontal regeneration is the use of an enamel matrix derivative (EMD).² The major components are amelogenins, a family of hydrophobic proteins derived from different splice variants and controlled post-secretory processing from a single gene that accounts for >95% of the total protein content.³ These proteins self-assemble into supramolecular aggregates that form an insoluble extracellular matrix that functions to control the ultrastructural organization of the developing enamel crystallites.³ Other proteins found in the enamel matrix include enamelin, ameloblastin (also called amelin or sheathlin), amelotin, apin, and various proteinases.⁴,⁵ The rationale for the clinical use of EMD is the observation that enamel matrix proteins (EMPs) are deposited onto the surface of developing tooth roots before cementum formation.⁶ Recent data from a systematic review indicate that EMD affects many different cell types, including epithelial cells, PDL cells, gingival fibroblasts, and osteoblasts.⁷

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It has been shown that EMD has effects on cell attachment, spreading, and chemotaxis; cell proliferation and survival; expression of growth factors, cytokines, and extracellular matrix molecules; and the expression of certain molecules that modulate bone remodeling.\textsuperscript{7} Histologic studies in animal and humans have provided evidence for periodontal regeneration after surgery with EMD.\textsuperscript{8-16} The application of EMD is usually performed after root surface conditioning with phosphoric acid, citric acid, or EDTA.\textsuperscript{17-21} In those clinical trials, the most frequently used root conditioning agent was 24% EDTA at neutral pH. The biologic advantages for using EDTA at neutral pH is based on findings from experimental studies indicating that, at neutral pH, EDTA appeared to effectively remove the smear layer produced by scaling and root planing, selectively remove smear layer and mineral from the dentin or cementum surface exposing a collagenous matrix, and produce a more biocompatible surface when compared to other root surface conditioning agents.\textsuperscript{22-28}

Although a plethora of research\textsuperscript{2,7} exists supporting the clinical use of EMD, it is currently unknown to what extent the bleeding that occurs during periodontal surgery may affect the adsorption of EMD to root surfaces. Therefore, the aims of this study are to: 1) test for EMD adsorption to the root surface in the presence of blood before and after conditioning with EDTA and coating with EMD; 2) determine the optimal procedure of coating EMD in a clinical setting by comparing six coating procedures; and 3) quantify the effect of surface coating of EMD on PDL cell attachment and proliferation in the presence of human blood.

**MATERIALS AND METHODS**

**Experimental Design**

Forty-six healthy human teeth extracted for orthodontic reasons were collected with written informed patient consent at the Department of Orthodontics, Dental Clinic, University of Bern, Bern, Switzerland. Human blood was obtained intravenously and coated on root surfaces after root scaling as illustrated in Figure 1. To determine the ability for EMD to adsorb to root surfaces, 24 human teeth were split into the eight experimental groups (performed in triplicate) and were fixed. Six clinically related coating procedures were tested as follows: 1) root scaling followed by application of EDTA\textsuperscript{§} for 2 minutes, rinsed with saline, followed by coating of EMD\textsuperscript{i} over blood for 5 minutes; 2) root scaling followed by application of EDTA for 2 minutes, rinsed with saline, followed by coating of EMD without blood for 5 minutes; 3) root scaling followed by application of EDTA for 2 minutes, rinsed with saline, dried, followed by coating of EMD without blood for 5 minutes; 4) root scaling followed by application of EDTA for 2 minutes with no additional application of EMD or blood (control); 5) root scaling with no application of EDTA, rinsed with saline, followed by coating of EMD over blood for 5 minutes; 6) root scaling with no application of EDTA, rinsed with saline, followed by coating of EMD without blood for 5 minutes; 7) root scaling with no application of EDTA, rinsed with saline, dried, followed by coating of EMD without blood for 5 minutes; and 8) root scaling with no application of EDTA, EMD, or blood (negative control).

**Scanning Electron Microscopy**

Treated root surfaces from the six experimental modalities were initially assessed by scanning electron microscopy (SEM). A seventh and eighth tooth were used as controls (extracted tooth receiving root scaling only and root scaling followed by treatment with EDTA alone). After root surface coating, teeth were fixed in 1% glutaraldehyde and 1% formaldehyde for 2 days. After serial dehydration with ethanol, samples were critical point dried.\textsuperscript{¶} The following day, samples were sputter coated using a sputtering device\textsuperscript{#} with 10 nm gold and analyzed microscopically to determine EMD protein adsorption to the root surface.

**Light Microscopic Immunohistochemistry**

To determine the ability for EMD to adsorb to root surfaces, 24 human teeth were split into the eight

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\textsuperscript{§} PrefGel, Institute Straumann, Basel, Switzerland.  
\textsuperscript{i}Emdogain, Institute Straumann.  
\textsuperscript{¶}Type M.9202 Critical Point Dryer, Roth & Co. Hatfield, PA.  
\textsuperscript{#}DCM-010, Balzers, Liechtenstein.
Experimental groups (three teeth per group) and were fixed in 4% formaldehyde for 2 days. An additional tooth not receiving any treatment option was used as a negative control. After decalcification in 4.13% disodium EDTA at 4°C for 6 weeks, the demineralized samples were washed in 0.1 M sodium cacodylate buffer containing 5% sucrose (pH 7.3) and placed in 70% ethanol overnight. The following day, samples were placed in 95% ethanol for 1 hour, isopropanol three times for 1 hour, xylene two times for 1 hour, and left overnight in xylene. The following morning, samples were placed three times in paraffin for 1 hour at 60°C and then processed for embedding in paraffin. Five-micrometer-thick sections were cut and incubated with an affinity-purified rabbit anti-EMD polyclonal antibody** at a dilution factor of 1:1000 using the streptavidin–biotin-labeled secondary antibody technique as described previously. After adding an equal amount of adjuvant, the solution was injected into a rabbit. The titer of the rabbit antibody against EMD was 1/10^6 (immunoglobulin G = 10 μg/L). Sections were counterstained with hematoxylin. Light microscopic recording was performed using a digital camera†† connected to a microscope.‡‡

Isolation of Human PDL Cells

PDL cells were obtained from three periodontally healthy patients in the Department of Orthodontics, Dental Clinic at the University of Bern after informed written consent. Briefly, extracted teeth were placed in Dulbecco’s modified Eagle medium with 10% fetal bovine serum and antibiotics (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B§§). PDL cells were harvested from the middle third portion, explanted in T-25 cell culture flasks, and grown to confluency. All cells during experimental seeding were from passage 3.

Adhesion and Proliferation Assays

Twenty-one teeth were cut in half longitudinally, placed into six-well culture plates, and treated similarly to a previous in vitro study on root surfaces. An additional control group receiving regular root planing with no additional blood, EDTA, or EMD was used for normalization purposes. PDL cells were seeded at a density of 50,000 cells/5 mL. Cell number was measured by staining cells for actin with phalloidin–fluorescein isothiocyanate (FITC)ii for 8 hours for cell adhesion and 5 days for cell proliferation assays. At each time point, teeth were removed from six-well dishes, transferred to new six-well dishes containing 4% formaldehyde for 15 minutes, and stained for phalloidin–FITC as described previously.

** Biora, Malmo, Sweden.
†† ProgRes C5 digital camera, Jenoptik Laser, Optik Systeme, Jena, Germany.
‡‡ Axioplan microscope, Carl Zeiss, Göttingen, Germany.
§§ Invitrogen, Basel, Switzerland.
ii Invitrogen.
Ten fields of view were captured per tooth and nuclei were counted using image analysis software. Three independent experiments were performed. Data were normalized to control, uncoated samples and analyzed for statistical significance using one-way analysis of variance with Bonferroni test.

RESULTS

SEM Coating of EMD
Root surfaces conditioned with EDTA, followed by blood + EMD, revealed a surface layer that was covered with a fibrin network from blood plasma proteins (Fig. 2A). Erythrocytes were visualized on the surface in all blood-coated samples (Fig. 2B). The microtopographies of blood + EMD-coated samples (Fig. 2C) revealed a more smooth microtopography when compared to EMD-coated samples (Figs. 2F and 2I). When human root surfaces were rinsed after EDTA conditioning, addition of EMD revealed substantial protein deposition on the root surface of human teeth (Figs. 2D and 2E). High-resolution images revealed a network of EMD protein fibers spanning over the entire surface (Fig. 2F). When root surfaces were dried after rinsing with saline, a very even, tightly-packed protein layer of EMD was visible on the root surface (Fig. 2G). The high-resolution images displayed similar microtopographies as those observed in rinsed + EMD samples (Figs. 2F and 2I). Root surfaces treated with EDTA and not receiving any additional blood or EMD were vacant of a visible layer of protein coating (Figs. 2J through 2L).

Root surfaces that were not coated with EDTA showed similar patterns of EMD adsorption (Fig. 3). Blood + EMD-coated surfaces displayed an even layer of blood plasma proteins that distributed across the root surface (Figs. 3A and 3B). These proteins seemed to coalesce into what appeared to be a much smoother microtopography compared to EMD-coated surfaces (Figs. 3C when compared to EMD-coated surfaces (Figs. 3F and 3I)). All surfaces that were rinsed with saline followed by EMD application revealed a root surface displaying EMD adsorption (Figs. 3D through 3F). When surfaces were dried, EMD adsorption appeared even over the entire root surface (Figs. 3G through 3I), with microtopographies similar to those observed in EDTA-treated surfaces (Fig. 2I). Control uncoated surfaces displayed a visible cementum layer with no apparent deposition of proteins after root scaling (J through L). (A, D, G, J = original magnification x200; B, E, H, K = x800; C, F, I, L = x6400).

Figure 3.
SEM images of root surfaces not treated with EDTA followed by blood + EMD (A through C), rinsed with saline, coated with EMD (D through F), and rinsed with saline, dried, coated with EMD (G through I). The blood-coated samples contained a very smooth surface mixed with blood plasma + EMD proteins when observed at high magnification (C). The EMD-coated surfaces display similar macrotopographies and nanotopographies as those observed in the EDTA-treated samples. The most even application of EMD was observed in the samples that were rinsed followed by drying before application of EMD (G through I). Control uncoated root surfaces displayed a visible cementum layer with no apparent deposition of proteins after root scaling (J through L). (A, D, G, J = original magnification x200; B, E, H, K = x800; C, F, I, L = x6400).

were captured using a microscope with a digital camera. Ten fields of view were captured per tooth and nuclei were counted using image analysis software. Three independent experiments were performed. Data were normalized to control, uncoated samples and analyzed for statistical significance using one-way analysis of variance with Bonferroni test.

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Olympus BX-51, Olympus, Center Valley, PA.

ProgRes CT3 digital camera, Jenoptik Laser, Optik Systeme.

NIH ImageJ, National Institutes of Health, Bethesda, MD.
Light Microscopic Immunohistochemistry
EMD was detected on the root surface of all treatment modalities (Fig. 4). For surfaces treated with EDTA for 2 minutes, followed by blood + EMD, EMD staining was lightly observed on the root surface (Fig. 4A). A positive but weak immunoreaction was also observed at the dentinocemental junction (Fig. 4A). At surfaces that were coated with EMD alone after EDTA application, EMD staining was observed on the root surface in a more consistent pattern (Fig. 4B). When the root surface was completely dried after application of EDTA and rinsing, intensely labeled EMD was observed on the surface in a consistently distributed pattern (Fig. 4C). When root surfaces were treated without EDTA, the blood + EMD groups showed little to no EMD staining (Fig. 4D). Application of EMD alone to the root surface rinsed with saline revealed once again an evenly coated surface irrespective of EDTA (Fig. 4E). Samples rinsed and dried and coated with EMD displayed intensely stained root surfaces for EMD (Fig. 4F). Remnant soft tissue on the root surface not removed by scaling was consistently intensely labeled with anti-EMD (data not shown).

PDL Cell Attachment and Proliferation Rates
PDL cells attached and proliferated on all surfaces. Root surfaces treated with EDTA did not appear to influence PDL cell attachment or proliferation (Fig. 5). Samples treated with EDTA, followed by coating with blood + EMD, displayed a relative attachment level of 94% control values (Fig. 5A). When samples were rinsed or rinsed + dried, followed by coating with EMD, PDL cell attachment improved to 110% and 120%, respectively. In samples not treated with EDTA, PDL cell attachment levels were quantified at 94%, 115%, and 117% for blood + EMD-coated, rinsed +

Figure 4.
Anti-EMD fluorescent labeling for root surfaces coated with EDTA, EMD, and/or blood. Extracted teeth from the dental clinic received root scaling followed by EDTA for 2 minutes, coated with EMD over blood (A); EDTA for 2 minutes, rinsed with saline, coated with EMD (B); EDTA for 2 minutes, rinsed with saline, dried, coated with EMD (C); coated with EMD over blood (D); rinsed with saline, coated with EMD (E); and rinsed with saline, dried, coated with EMD (F). Root surfaces that came into contact with blood showed a thin layer of EMD coated on the root surface when coated with EDTA (A) and minor to no staining without application of EDTA (D). When blood was not present on the root surface, EMD was able to evenly adsorb to the root surface (B and E) with the best outcomes observed in rinsed + dried samples (C and F). D = dentin; DCJ = dentinocemental junction; AEFC = acellular extrinsic fiber cementum. Scale bar = 20 μm.
Enamel Matrix Protein Adsorption to Root Surfaces

We found that EMD remained bound to extracted human teeth treated with EMD 4 weeks after application.29 It appears that having EMD properly adsorbed to root surfaces could alter clinical outcomes. The impact of having other proteins, such as those found in blood plasma, competing with EMD adsorption to root surfaces and alter PDL cell attachment and proliferation at later time points. It has been shown previously that EMD adsorbs to hydroxyapatite, collagen, and denuded tooth roots in which it persists for up to 2 weeks after surgery as an insoluble complex.32 Furthermore, experiments using immunohistochemical analysis found that EMD remained bound to extracted human teeth treated with EMD 4 weeks after application.29

It appears that having EMD properly adsorbed to root surfaces could alter clinical outcomes. The impact of having other proteins, such as those found in blood plasma, competing with EMD could cause unspecific binding of unwanted proteins. SEM analysis revealed many fibrin networks with erythrocytes and blood plasma proteins in root surfaces coated with blood + EMD. This altered the final nanotopography of EMPs when compared to root surfaces coated with EMD in the absence of blood (Figs. 2C and 2F). Interestingly, it has been shown previously that EMPs bind to wound matrix proteins, such as fibronectin, laminin-1, and collagen types I and IV.33 Narani et al. found that collagen and fibronectin significantly reduces binding of EMPs alone.33 Plasma fibronectin is a major protein component of the blood plasma and has a circulatory concentration of 300 μg/mL. In our study, blood + EMD-coated root surfaces reduced PDL cell attachment by ≤25% irrespective of EDTA root conditioning (Fig. 5A) and significantly reduced PDL cell proliferation by 30% (Fig. 5B). The major component of EMD, amelogenins, is a family of hydrophobic proteins that account for >90% of the total protein content and has been described previously as a cell adhesion molecule.32,34 Maximizing proper EMD adsorption to root surfaces during periodontal surgeries would likely lead to improved clinical outcomes.

We also addressed the role of EDTA root conditioning on the ability for EMD to adsorb to root surfaces and affect PDL cell attachment and proliferation. SEM analysis did not demonstrate noticeable differences in the ability for EMD to adsorb and cover the root surface with EDTA. Immunohistochemistry of root surfaces treated with versus without EDTA also showed very similar patterns of EMD adsorption (Fig. 4). One difference observed in the EMD-coated root surfaces was the ability for EMD to penetrate the surface of non-EDTA-treated modalities when compared to EDTA-treated root surfaces (Figs. 4C and 4F). In EDTA-treated modalities, the ability for EDTA to generate an even layer of demineralized acellular extrinsic fiber cementum matrix allowed a more even labeling for EMD at the root surface (Fig. 4C). In contrast, root surfaces lacking EDTA treatment did not decalcify the rugged or jagged surface structure created during scaling, and the microcracks enabled EMD to preferentially penetrate between microcracks (palisade-like labeling pattern seen in Fig. 4F). Crack formation may be related to the presence and arrangement of Sharpey’s fibers. The effect of EDTA root conditioning on the healing of intrabony defects treated with an EMP derivative has been clinically tested.21,35 No statistical differences were observed between open-flap debridement (OFD) followed by root surface conditioning with EDTA and application of EMD versus that of OFD + EMD alone.21,35 Nevertheless, the choice of EDTA for root surface conditioning was based on results of previous histologic studies in animals that provided evidence that application of EDTA significantly enhanced periodontal wound healing compared to other conditioning agents.

**DISCUSSION**

The results from the present study demonstrate that bleeding, which occurs during periodontal surgery, may compete with EMD adsorption to root surfaces and alter PDL cell attachment and proliferation at later time points. It has been shown previously that EMD adsorbs to hydroxyapatite, collagen, and denuded tooth roots in which it persists for up to 2 weeks after surgery as an insoluble complex.32 Furthermore, experiments using immunohistochemical analysis found that EMD remained bound to extracted human teeth treated with EMD 4 weeks after application.29

EDM-coated, and rinsed, dried + EMD-coated root surfaces, respectively (Fig. 5A). PDL proliferation displayed similar trends with significantly higher proliferation rates were observed in all EMD-coated samples when compared to blood + EMD-coated samples (Fig. 5B). All samples displayed higher proliferation rates than control, uncoated samples. The highest proliferation rates were observed in samples that were rinsed with saline, dried, followed by coating with EMD irrespective of EDTA (135%) versus without (132.3%) (Fig. 5B).

**Figure 5.**

Relative adhesion (A) and proliferation (B) rates for PDL cells seeded on root surfaces treated in six clinical settings normalized to control uncoated root surfaces. A) Higher adhesion values were observed on EMD-coated surfaces that were not mixed with blood. Both blood + EMD groups (either with or without EDTA) displayed lower adhesion values than control, uncoated surfaces. * P < 0.05.

PDL cells by ≤50% when compared to EMPs alone.33 Plasma fibronectin is a major protein component of the blood plasma and has a circulatory concentration of 300 μg/mL. In our study, blood + EMD-coated root surfaces reduced PDL cell attachment by ≤25% irrespective of EDTA root conditioning (Fig. 5A) and significantly reduced PDL cell proliferation by 30% (Fig. 5B). The major component of EMD, amelogenins, is a family of hydrophobic proteins that account for >90% of the total protein content and has been described previously as a cell adhesion molecule.32,34 Maximizing proper EMD adsorption to root surfaces during periodontal surgeries would likely lead to improved clinical outcomes.

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such as phosphoric acid or citric acid.23,24,36 Conversely, previous results from controlled clinical studies evaluating the effects of root conditioning using EDTA gel as an adjunct to surgical therapy for the treatment of intraosseous periodontal defects have failed to show statistically significant differences in the treatment outcome among the groups treated with OFD + EDTA or OFD alone.28,37

In the present study, the variability of EMD adsorption to root surfaces observed in the SEM and immunohistochemistry created by blood interactions with EMD negatively affects PDL cell attachment and proliferation. This variability could be extremely vital during regenerative surgery in which the application of EMD to the root surface is highly susceptible to blood interactions. Thus, the removal of all remnants of blood after root scaling may be advised to maximize the ability for EMD to properly adsorb to the root surface. The results from the present study also suggest that the combination of rinsing with saline followed by drying of the root surface maximizes the ability for EMD to adsorb to root surfaces irrespective of EDTA.

CONCLUSIONS
Within their limits, the present findings indicate that minimizing root surface exposure to blood during periodontal surgery is advised to allow more complete EMD protein adsorption to root surfaces. Furthermore, results from these in vitro experiments support the clinical evidence that EMD is capable of periodontal regeneration by improving PDL cell attachment and proliferation to root surfaces of human teeth.

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